

Mol Genet Genomics (2002) 267: 124–132  
DOI 10.1007/s00438-002-0654-9

## ORIGINAL PAPER

## BEST AVAILABLE COPY

B. Seiboth · G. Hofmann · C.P. Kubicek

## Lactose metabolism and cellulase production in *Hypocrea jecorina*: the *gal7* gene, encoding galactose-1-phosphate uridylyltransferase, is essential for growth on galactose but not for cellulase induction

Received: 4 October 2001 / Accepted: 30 January 2002 / Published online: 22 February 2002  
© Springer-Verlag 2002

**Abstract** Lactose is at present the only soluble carbon source which can be used economically for the production by *Hypocrea jecorina* (= *Trichoderma reesei*) of cellulases or heterologous proteins under the control of cellulase expression signals. However, the mechanism by which lactose triggers the formation of cellulases is unknown. To enhance our understanding of lactose metabolism and its relationship to cellulase formation, we have cloned and characterized the *gal7* gene (for galactose-1-phosphate uridylyltransferase) of *H. jecorina*. The gene encodes a polypeptide of 43.8 kDa, the sequence of which exhibits a moderate level of identity (about 50%) to that of the Gal7 proteins of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*, and contains an active-site signature typical for galactose-1-phosphate uridylyltransferase family 1. *H. jecorina gal7* is not clustered with other genes of galactose metabolism. A single 1.7-kb transcript is synthesized constitutively during the rapid growth phase and accumulated to twice this level during incubation in the presence of D-galactose and L-arabinose and the corresponding polyols (dulcitol, arabinol). A *gal7* deletion mutant, constructed by replacing the *gal7* reading frame by the *H. jecorina pyr4* gene, was unable to grow on D-galactose between pH 4.5 and 7.5, thus proving that in *H. jecorina gal7* is essential for metabolism of D-galactose, whereas the growth rate of the mutant on lactose was only reduced by about 50%. The rate of formation of cellobiohydrolase Cel7A and the

abundance of the corresponding (*cbh1*) transcript during growth on lactose was only slightly lower in the absence of *gal7*, but a significant delay in decay of the *cbh1* transcript was noted during later stages of growth. The results suggest that *H. jecorina* uses only the Leloir pathway for metabolism of D-galactose and lactose. Furthermore, we conclude that metabolism of lactose past the galactose-1-phosphate step is not essential for cellulase formation.

**Keywords** Leloir pathway · Gene regulation · *Trichoderma* · Lactose · Filamentous fungi

### Introduction

The disaccharide lactose (1,4- $\beta$ -D-galactopyranosyl-D-glucose) is a byproduct of cheese production and thus a renewable carbon source which accumulates to amounts of 300,000 tons per year worldwide, of which 15% is used as a carbon source for various microbial fermentations (Roelfsema et al. 1990). For cellulase production by the ascomycete *Hypocrea jecorina* (anamorph: *Trichoderma reesei*), it represents virtually the only soluble carbon source which is economically feasible on a technical scale (Persson et al. 1991), and which is required exclusively for the use of *H. jecorina* to produce heterologous proteins under the control of cellulase promoters (Penttilä 1998). However, lactose is metabolized slowly, and cellulase yields on lactose-containing media are lower than on cellulose (Andrcotti et al. 1980). A knowledge of the rate-limiting steps in lactose metabolism in *H. jecorina*, and of the mechanisms by which the disaccharide triggers cellulase formation would thus help to improve the use of lactose as a carbon source. However, lactose metabolism has not yet been studied in *H. jecorina*, and molecular genetic information on lactose utilization is so far not available for filamentous fungi in general.

The biochemistry and genetics of lactose and galactose metabolism in fungi has been studied extensively in

Communicated by C. A. M. J. J. van den Hondel

B. Seiboth (✉) · G. Hofmann<sup>1</sup> · C.P. Kubicek  
Bereich Mikrobielle Biochemie und Gentechnologie,  
Institut für Biochemische Technologie und Mikrobiologie,  
Technische Universität Wien, Getreidemarkt 9,  
A-1060 Wien, Austria  
E-mail: bseiboth@mail.zserv.tuwien.ac.at  
Fax: +43-1-5816266

<sup>1</sup>Present address:

<sup>1</sup>Department of Biotechnology,  
Technical University of Denmark, Lyngby,  
DK-2800, Denmark

the yeasts *Kluyveromyces lactis* (Dickson and Riley 1989) and *Saccharomyces cerevisiae* (Fukasawa and Nogi 1989; Lohr et al. 1995). While the glucose moiety of lactose directly enters the glycolytic pathway, galactose has to be converted to glucose-6-phosphate, by a series of enzymatic reactions known as the Leloir pathway, in order to enter the glycolytic pathway. Both yeasts use the Leloir pathway exclusively for this process (Frey 1996). In contrast, plants use an alternative pathway (Gross and Phar 1982; Gross and Schnarrenberger 1995). In the filamentous fungus *Aspergillus nidulans*, the operation of an alternative pathway during growth at pH values above 7.5 has been suggested (Roberts 1970), but it is not known whether this is the same pathway as the one proposed to occur in plants.

Galactose-1-phosphate uridylyltransferase (UDP-glucose: $\alpha$ -D-galactose-1-phosphate uridylyltransferase; EC 2.7.7.12), which catalyzes the reversible transfer of the uridine 5' phosphoryl moiety from UDP-glucose to galactose-1-phosphate to produce UDP-galactose and glucose-1-phosphate, is a key enzyme of the Leloir pathway. As a first step towards the characterization of lactose metabolism in *H. jecorina* and its relationship to cellulase formation, we report here the cloning and functional characterization of the gene encoding UDP-glucose: $\alpha$ -D-galactose-1-phosphate uridylyltransferase (*gal7*). We will provide evidence that this gene product is essential for growth on galactose, but not for induction of cellulase formation on lactose.

## Materials and methods

### Strains and culture conditions

The *H. jecorina* strains used in this study were QM9414 (ATCC 26921) and the *pyr4* negative mutant TU-6 (ATCC MYA-256, Gruber et al. 1990a). All strains were maintained on malt-extract agar, supplemented with uridine (10 mM) when necessary, and grown in 250-ml flasks on a rotary shaker (250 rpm) at 30°C in the minimal medium described by Mandels and Androoti (1978) with the respective carbon source at a final concentration of 10 g/l.

For the analysis of cellulase expression, strains QM9414 and  $\Delta$ Gal7 were grown on glycerol (1% w/v) for 24 h; mycelia were harvested by filtration and washed with tap water; then equal amounts of mycelia were transferred to flasks containing lactose (1% w/v) as a carbon source, and cultivation was continued for 84 h. The expression of *gal7* was analyzed in the same way, but using cultures that had first been grown for 24 h on glycerol (1% w/v), and then transferred into a medium containing the carbon source of interest for a further incubation for 6 h. In the case of pectin, arabinogalactan and lactose, *gal7* expression was analyzed without initial growth on glycerol.

The *Escherichia coli* strains ER1647 and BM25.8 (Novagen, Madison, Wis.) were used for library screening, and strain JM109 (Promega, Madison, Wis.) for plasmid propagation.

### Cloning of the *H. jecorina gal7* gene

To amplify a potential *H. jecorina gal7* fragment by PCR, the conserved amino acid sequences QIFENKGTAM and DITPEQAA were chosen as the basis for the design of oligonucleotide primers. The degenerate primers *udgtfor1* (5'-CA(A/G)AT(CT)TT(CT)GA(A/G)AA(CT)AA(A/G)GGIACIGCIATG-3') and *udgt-*

*rev1* (5'-GCIGC(CT)TG(CT)TCIGGIGT(A/G)AT(A/G)TC-3') were used, together with 100 ng of *H. jecorina* QM9414 genomic DNA as template, in a 50- $\mu$ l reaction volume incubated in an automated temperature cycling device (Biotron, Biometra, Göttingen, Germany). The reaction mixture contained, in addition, 2.5 mM MgCl<sub>2</sub>, 10 mM TRIS-HCl pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100, 0.4  $\mu$ M of each primer, 0.2 mM of each dNTP and 0.5 U of *Taq* polymerase (Promega). After initial denaturation at 94°C for 1 min, 30 cycles of amplification were carried out (1 min at 94°C, 1 min at 55°C, 1 min at 74°C), with a final extension period of 7 min at 74°C. A 550-bp fragment was isolated and cloned into pGEM-T (Promega).

The 550-bp fragment was used to screen a genomic library of *H. jecorina* QM 9414 in  $\lambda$ BlueSTAR (Novagen). The *gal7* gene was localized on a 3.5-kb *NsiI*-*EcoRV* fragment which was ligated into pGEM-5Zf(+), resulting in the plasmid pGAL7. Restriction fragments from various chromosomal subclones were ligated into pBluescript SK(+) and sequenced, using a Li-Cor 4200 (Li-Cor, Lincoln, Neb.) automatic sequencer. The assembled DNA sequence was deposited in GenBank under Accession No. AY057108.

For the cloning of a *gal7* cDNA fragment comprising the entire coding region, poly(A)<sup>+</sup> mRNA from galactose-grown mycelia was isolated with the aid of the PolyAtract mRNA Isolation System (Promega). The *gal7* cDNA was then amplified by RT-PCR using the Reverse Transcription System (Promega) with the primer pair *gal7for* (5'-CCGATATCATGCCTGACAAGATCCTC-GATG-3') and *gal7rev* (5'-GTCTAGCTCAACTGTTCGG-3').

### Sequence analysis

The 3.5-kb fragment was analyzed using BLAST programs (Altschul et al. 1990) and multiple sequence alignment was done using MultiAlin (Corpet 1988). Consensus binding sequences in the *gal7* 5' region were identified manually.

### Construction of a *H. jecorina* $\Delta$ gal7 strain

To construct a  $\Delta$ gal7 strain of *H. jecorina*, the *gal7* coding region in pGAL7 was replaced by the *H. jecorina pyr4* marker. To this end, the entire *gal7* coding region was deleted using a two-step fusion-PCR approach (Higuchi 1990). The primers used for amplification of the 5' and 3' untranslated regions were the universal primers M13f and M13r, and *gal7del1* (5'-GAGATGTCTAGCTCGTCGACGGTAGGGAATTATCCTGGTG-3') and *gal7del2* (5'-GATAATTCCCTACCGTCGACGAGCT AGACATCTCAGCC T-3'). The resulting 2.1-kb fragment comprising the 5' and 3' flanking regions without the *gal7* coding region was ligated into the *NsiI*/*EcoRV* sites of pGEM-5Zf(+). The recombinant PCR introduced a single *SalI* site between the 5' and 3' *gal7* flanking regions, which was used to introduce a 2.7-kb *pyr4 SalI* fragment of *H. jecorina* from pFG1 (Gruber et al. 1990b), resulting in the plasmid pDGAL7.

### Fungal transformation

Transformation of *H. jecorina* TU-6 was done according to Gruber et al. (1990b). For gene replacement pDGAL7 was linearized with *NsiI* and *EcoRV*, and the resulting 4.8-kb fragment isolated from an agarose gel.

### Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with tap water, then frozen and ground in liquid nitrogen. For extraction of DNA, mycelial powder was suspended in buffer A (1.2 M NaCl, 5 mM EDTA, 0.1 M TRIS-HCl, pH 8.0), incubated for 20 min at 65°C, cooled on ice, mixed with 1 vol of phenol and 1 vol of chloroform and centrifuged (12,000 rpm, 15 min) in an Eppendorf centrifuge. Following a second extraction with 1 vol of chloroform, the DNA

126

was precipitated with 1 vol of isopropanol and washed with 70% (v/v) ethanol. Total RNA was isolated as described by Chomczynski and Sacchi (1987). Standard methods (Sambrook et al. 1989) were used for electrophoresis, blotting and hybridization of nucleic acids.

The probes used for hybridization were: a 1.4-kb *Bgl*I fragment of *cbh1* (the gene encoding cellobiohydrolase Cel7A), a 1.9-kb *Acc*65I fragment of the *act1* (actin) gene, and a 2.4-kb *Bst*XI fragment of *gal7*. Signal intensities were determined by densitometric measurements of autoradiographs exposed for various times [only values with linear correlation ( $r > 0.9$ ) were used].

#### Determination of fungal growth

To determine rates of hyphal growth on agar plates, plates were inoculated by placing a small piece of mycelium-bearing agar in the center of an 11-cm plate, and the increase in colony diameter measured twice daily. To measure growth in submerged cultures, the increase in biomass dry weight was recorded. To this end, mycelia were harvested after appropriate times, washed extensively with distilled water and dried to constant weight.

#### Biochemical analysis

Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany). The protocols described by Ausubel et al. (1991) were used for SDS-PAGE (using 10% polyacrylamide gels), and Western blotting (using a monoclonal antibody to detect cellobiohydrolase Cel7A; Mischak et al. 1989).

## Results

#### Cloning of a *H. jecorina* galactose-1-phosphate uridylyltransferase

We identified conserved amino acid regions in various Gal7 proteins by aligning the corresponding amino acid sequences from *S. cerevisiae*, *K. lactis*, *Filobasidiella neoformans* (*Cryptococcus neoformans*), *Homo sapiens* and *E. coli*. Gene-specific degenerate primers derived from these regions were successfully used to amplify a single 550-bp amplicon; the polypeptide encoded by this fragment exhibited a high degree of identity to other galactose-1-phosphate uridylyltransferases. With this amplicon as a probe, a 3.5-kb genomic clone was isolated from a *H. jecorina* library in  $\lambda$ BlueSTAR and sequenced. This clone contained an ORF of 1353 bp, interrupted by three introns of 74, 67 and 66 bp, respectively, the positions of which were verified by sequencing a corresponding cDNA clone. Southern analysis of differentially digested chromosomal DNA suggests that *gal7* is present in a single copy in the *H. jecorina* genome (data not shown).

The deduced polypeptide comprises 382 residues with a calculated  $M_r$  of 43.8 kDa, which shows moderate identity over its entire sequence to Gal7p from yeasts (*S. cerevisiae*, 51% identity; *K. lactis*, 53% identity; *F. neoformans*, 42% identity), mammals (*H. sapiens*, 42% identity) and prokaryotes (*E. coli*, 47% identity). Moreover, single amino acids and sequence motifs which have already been shown to be important for the

function of the *E. coli* GalT (highlighted in Fig. 1), such as the consensus sequence of the active site (GCSNPHPHGQ; Wedekind et al. 1996), and the amino acids involved in binding zinc ( $C_{52}$ ,  $C_{53}$  (the rubredoxin "knuckle" motif),  $H_{115}$  and  $H_{164}$ ) and iron ( $H_{281}$ ,  $H_{296}$ ,  $H_{298}$  and  $E_{182}$ ; Ruzicka et al. 1995; Wedekind et al. 1995) are conserved. Thus, *H. jecorina gal7* encodes a type-1 galactose-1-phosphate uridylyltransferase with high functional similarity to the enzyme from bacteria and yeast, but unlike that of mammalian cells and red algae, in which three of the four residues involved in zinc binding are non-conservatively replaced (Wedekind et al. 1995; Lluisma and Ragan 1998).

#### *H. jecorina gal7* is not clustered with other *gal* genes

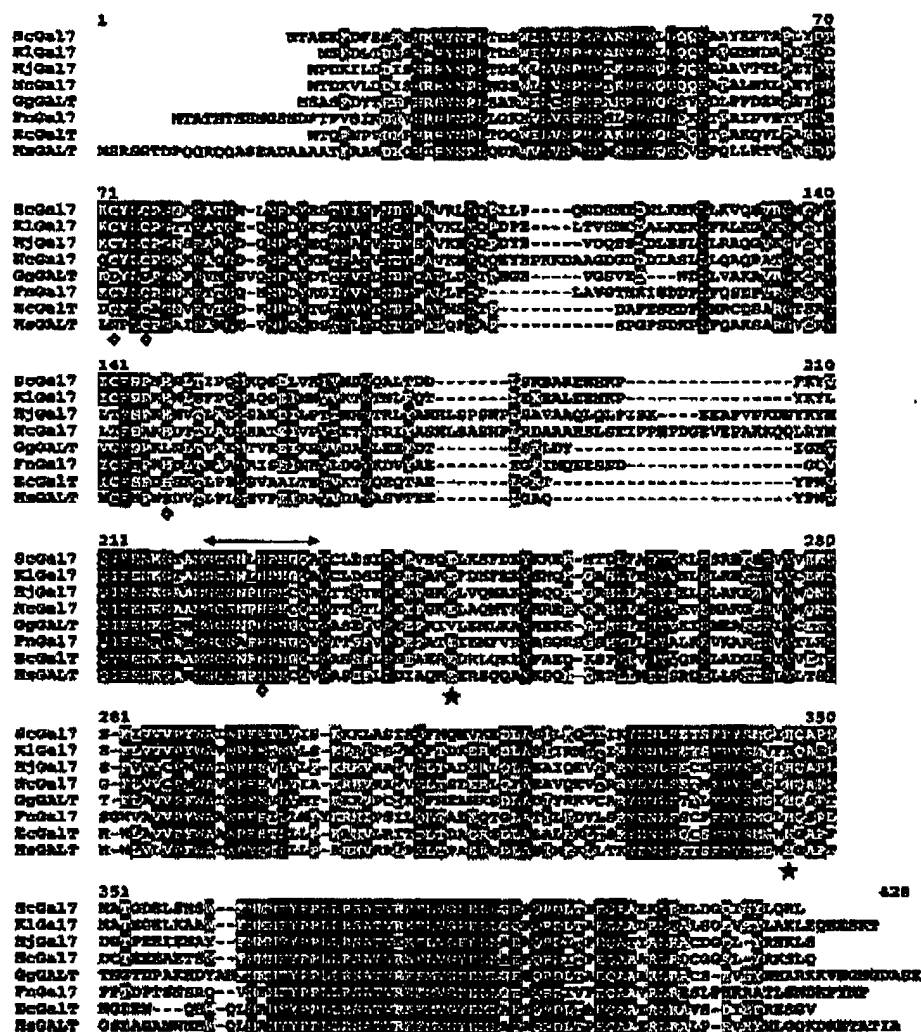
The genes encoding the enzymes of the Leloir pathway – *GAL1* (encoding the galactokinase), *GAL7* and *GAL10* (UDP-glucose 4-epimerase) – are organized as a cluster in yeasts such as *S. cerevisiae* and *K. lactis* (Dickson and Riley 1989; Fukasawa and Nogi 1989; Lohr et al. 1995). With *H. jecorina gal7* in hand, we asked whether such a *gal* gene cluster is also present in this fungus. To this end, we performed a chromosome walk from the 5' and 3' flanking regions of *gal7* until we encountered other ORFs (Fig. 2). In the region 5' to *gal7* we discovered a gene which codes for a protein that shows high similarity (sequence identity 53%) to *S. cerevisiae* Ada2p, whereas the next ORF downstream of *gal7* codes for a protein of as yet unknown function, that displays a high level of similarity (sequence identity 47%) to that encoded by an ORF in the *Neurospora* genome database (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora>; contig 1.121, bp 12336–12839); and moderate similarity (sequence identity 31%) to *S. cerevisiae* Erg28p (Goffeau et al. 1996). Hybridization analysis with PCR fragments encoding the *H. jecorina GAL1* and *GAL10* homologs (B. Seiboth, unpublished data) showed that these two genes are not located within an area extending approximately 9 kb up- and downstream of *gal7*. From these data, we conclude that in *H. jecorina*, unlike the case in yeasts, *gal7* is not clustered with genes for other enzymes of the Leloir pathway.

#### Regulation of *gal7* expression

In the 5' upstream sequences of *H. jecorina gal7*, consensus binding sites for several known proteins were identified: A binding site for the carbon catabolite repressor Cre1 (Strauss et al. 1995; Ilmen et al. 1996) is found at –47, for XlnR, the *A. niger* transcriptional activator of xylanase and cellulase biosynthesis (van Peij et al. 1998) at –153 and –207; for PacC, the *A. nidulans* pH regulator (Denison 2000) at –383; and a site for Hap2/3/5, a multimeric complex that binds to CCAAT motifs (Brakhage et al. 1999; Zeilinger et al. 2001) is located at –368 (Fig. 3A). Interestingly, no sequences

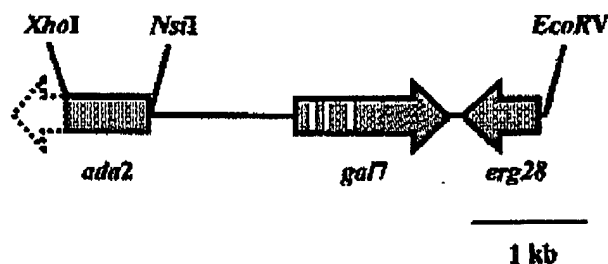
127

**Fig. 1** Alignment of galactose-1-phosphate uridylyltransferases. ScGal7 (*Saccharomyces cerevisiae*, P084313), KlGal7 (*Kluyveromyces fragilis*, P09580), HjGal7 (*Hypocrea jecorina*, AY057108), NcGal7 (*Neurospora crassa*; <http://www-genome.wi.mit.edu/annotation/fungi/neurospora; contig.1.121, bp 10732-12109>), GgGALT (*Gracilaria gracilis*, AAB88705), FnGal7 (*Filobasidiella neofornans*, S69795), EcGalT (*Escherichia coli*, P09148), HsGALT (*Homo sapiens*, P07902). Residues in white on a black background are conserved in at least 90% of the Gal proteins, residues in white on gray in 40%. The double-headed arrow indicates the position of the active site, residues involved in zinc binding are designated by diamonds and amino acids required for iron binding are marked by stars (see text)



matching the consensus for binding of the transcriptional activators of the genes of the Leloir pathway in *S. cerevisiae* (Gal4p) and *K. lactis* (Lac9p) (CGGN<sub>11</sub>CCG; Dhawal and Lane 1993) were found.

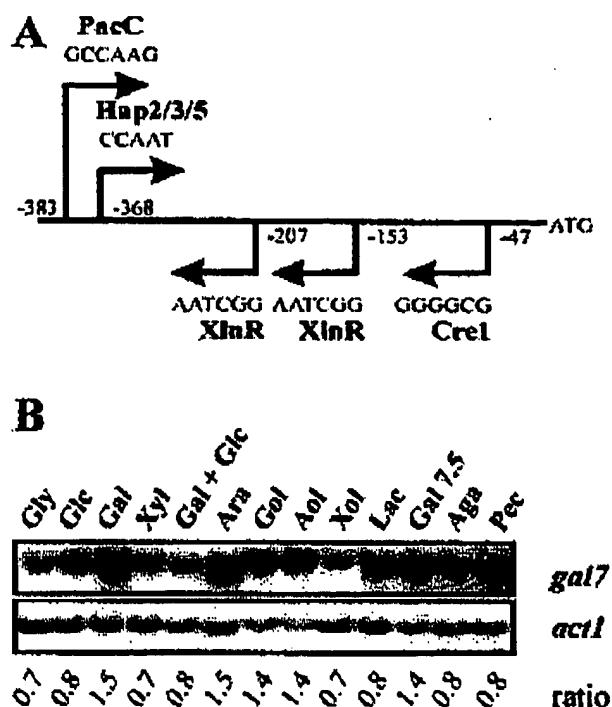
The presence of the above-mentioned consensus sequences prompted us to investigate whether *gal7* expression might be regulated by carbon catabolite repression, pH, or by the presence of xylose. To test this, we measured the abundance of the *gal7* transcript by Northern analyses during growth of *H. jecorina* on a variety of carbon sources. Generally, the *gal7* transcript was most abundant in young cultures, and levels decreased sharply during further cultivation (data not shown). To minimize differences in growth rate on the carbon sources investigated, *H. jecorina* was first cultured on glycerol. Only in the case of lactose, and the polymers arabinogalactan and pectin, was this preculture step omitted. However, Fig. 3 shows that accumulation of a 1.7-kb *gal7*



**Fig. 2** Genomic organization of the *gal7* locus in *H. jecorina*. The arrows indicate the orientation of the ORFs and the white boxes show the positions of the introns in the *gal7* gene

transcript was detected on essentially all carbon sources tested. Increased transcript levels (approximately twofold compared to the level on all other carbon sources

128



**Fig. 3A, B Regulation of *gal7* expression in *H. jecorina*.** **A** Positions of consensus binding sites for known fungal proteins in the non-coding region upstream of *gal7*. Positions are given relative to the translation start codon ATG. The arrows indicate the orientation of the consensus sequences on the coding strand (arrow above) and non-coding strand (arrow below). **B** Northern analysis of the *gal7* transcript during growth on various carbon sources. Mycelia were cultured at pH 4.5, and in the case of galactose also at pH 7.5 (Gal7.5). Samples were obtained 6 h after transfer to the respective carbon source. In the case of lactose, arabinogalactan and pectin, the QM9414 strain was cultivated without prior growth on glycerol, and samples were taken after 30 h of growth. The values indicate the ratio of the intensity of the *gal7* signal (measured by densitometry) to that of the *act1* control. Abbreviations: Gly, glycerol; Glc, D-glucose; Gal, D-galactose; Xyl, xylose; Ara, L-arabinose; Gol, galactitol; Aol, L-arabitol; Xol, xylositol; Lac, lactose; Aga, arabinogalactan; Pec, pectin

investigated) were detected on D-galactose and L-arabinose, and the corresponding polyols galactitol and arabitol. The *gal7* transcript was equally abundant at pH 4.5 and 7.5. Simultaneous incubation with D-glucose and D-galactose did not give rise to any increase in *gal7* transcript abundance, indicating that D-glucose interferes with transcriptional stimulation by D-galactose, thereby explaining why only constitutive levels of the transcript were observed on lactose.

The *gal7* gene is necessary for growth on galactose but not on lactose

In order to investigate whether the Leloir pathway is essential for growth of *H. jecorina* on D-galactose, we constructed a  $\Delta gal7$  mutant by replacing the coding

region of *gal7* by the *H. jecorina pyr4* gene (Fig. 4A). The corresponding mutant strain displayed similar growth rates on glucose and glycerol to the wild-type strain, but failed to grow on D-galactose. Interestingly, the mutant could grow on lactose, albeit at a reduced rate (Fig. 4B). This indicates that hydrolysis of lactose is not significantly impaired in the mutant, and is thus independent of the metabolites that arise downstream of the galactose-1-phosphate uridylyltransferase step. Roberts (1963) reported that in *A. nidulans* an alternative pathway accounts for D-galactose metabolism at pH values above 7.5. To find out whether such an alternative pathway might also exist in *H. jecorina*, we examined the effect of pH on the growth of wild-type *H. jecorina* and the  $\Delta gal7$  mutant on galactose. While the wild-type grew very poorly on D-galactose at pH 7.5, the  $\Delta gal7$  mutant of *H. jecorina* was virtually unable to grow under these conditions (Fig. 4C), thus arguing against the presence of such an alternative pathway in this fungus.

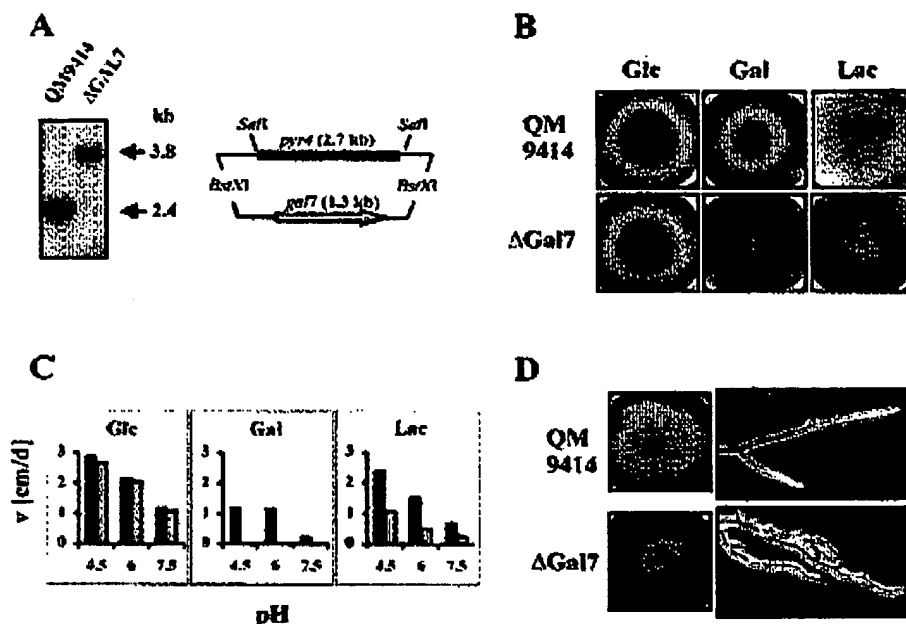
Loss-of-function of galactose-1-phosphate uridylyltransferase is known to lead to galactose toxicity in mammalian cells (Leslie et al. 1996; Ning et al. 2000). We therefore tested whether the *H. jecorina*  $\Delta gal7$  strain could grow on glucose in the presence of galactose. Figure 4d shows that the strain could indeed grow, but did so at about half the rate of the parent strain, and with different morphology. Microscopic examination revealed that the hyphae were more curved, partially swollen and characterized by thicker cell walls than the parent strain. Thus, galactose appears to affect hyphal growth of a *gal7*-deficient strain on glucose.

#### Effect of interruption of the Leloir pathway on the formation of Cel7A and *cbh1* gene expression in *H. jecorina*

To determine if metabolism of the D-galactose moiety of lactose is essential for cellulase production and cellulase gene transcription, *H. jecorina* QM 9414 and the  $\Delta gal7$  mutant were grown on lactose, and the level of the major cellulase protein cellobiohydrolase Cel7A (formerly referred to as CBH I) and the accumulation of the corresponding transcript were measured. The concentration of Cel7A in the medium was lower in the mutant, but so was also the biomass density and the amounts of Cel7A formed per gram of biomass were roughly similar in the wild-type strain and the  $\Delta gal7$  strain (Fig. 5A, B). This finding is supported by the fact that the abundance of the *cbh1* transcript was virtually the same in both strains (Fig. 5C). However, the decay in transcript abundance, which occurs during prolonged cultivation, was strongly reduced in the  $\Delta gal7$  mutant.

#### Discussion

The mechanisms governing expression of the *GAL* genes in *S. cerevisiae* have become a paradigm for gene



**Fig. 4A–D** Effect of *gal7* gene disruption on growth of *H. jecorina* on various carbon sources. **A** Southern analysis and gene deletion strategy. Genomic DNA of strain QM9414 and the  $\Delta gal7$  strain were digested with *BstXI* and probed with a 2.4-kb *BstXI* fragment comprising the coding as well as 5' and 3' UTRs of the *gal7* gene. On the right is a restriction map of *gal7* and the *gal7* deletion cassette. **B** Comparison of the growth of the wild-type strain QM9414 and the  $\Delta gal7$  strain on plates containing glucose, galactose or lactose as sole carbon source. **C** Effect of carbon source and pH on the radial growth rate (in cm per day) of *H. jecorina* QM 9414 (filled bars) and the corresponding  $\Delta gal7$  strain (stippled bars). **D** Comparison of the morphology of *H. jecorina* strain QM9414 and the  $\Delta gal7$  mutant strain during growth on 1% (w/v) glucose and 1% galactose

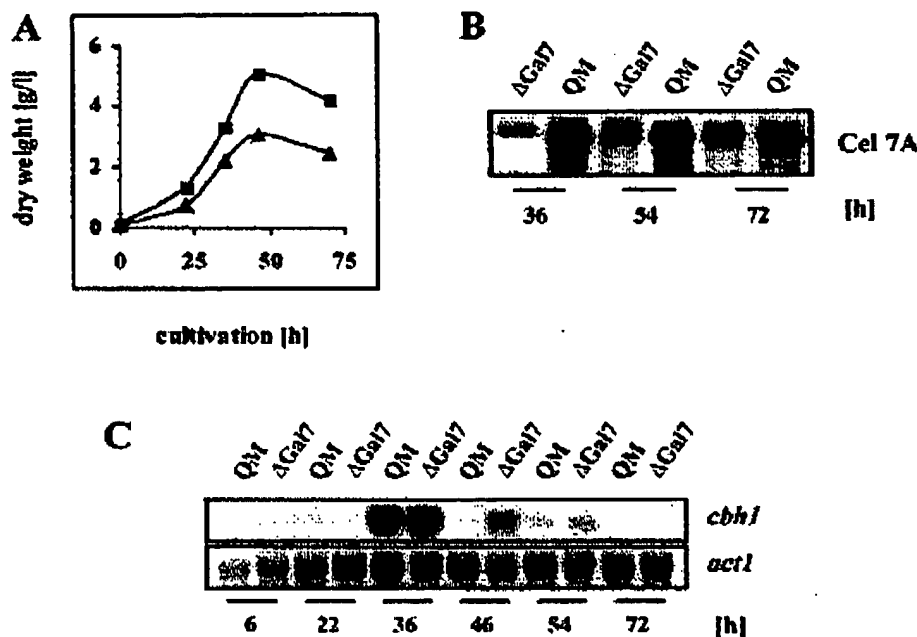
regulation in lower eukaryotes (Lohr et al. 1995). Largely similar results have been reported for the closely related yeast *K. lactis* (Dickson and Riley 1989) and for *gal7* expression in the fungal pathogen *Filobasidiella neoformans* (Wickes and Edman 1995). The present study is the first reporting an investigation of galactose metabolism in a filamentous fungus at the molecular genetic level. Although the deduced amino acid sequence of the *H. jecorina gal7* gene product is reasonably similar to those of *S. cerevisiae* and *K. lactis*, supporting the assumption that the enzyme has similar properties to the yeast galactose-1-phosphate uridylyltransferases, important differences relative to *GAL7* from *S. cerevisiae* and *K. lactis* were found with respect to genomic environment and regulation of expression. With respect to the former, we clearly showed that *gal7* is not found in a cluster with the other *H. jecorina gal* genes. In addition, we have evidence that *gal1* and *gal10* (encoding galactose kinase and UDP-glucose 4-epimerase, respectively) do not form a cluster (B. Seiboth, unpublished data). Thus, although clustering of genes is not

uncommon in filamentous fungi, and has frequently been found for genes involved in secondary metabolism (Smith et al. 1990; Keller and Adams 1995; Tsai et al. 1999) and assimilation of nitrogen and sulfur compounds (Johnstone et al. 1990; Schicova et al. 2000), it does not occur with the *H. jecorina gal* genes.

In *S. cerevisiae*, the *GAL* structural genes *GAL1*, *GAL7* and *GAL10* can be found in three major types of regulated states: inactive-repressed (glucose), inactive but poised for induction (glycerol), and induced (galactose) (Lohr et al. 1995). In contrast, *H. jecorina gal7* is not inactive in the presence of glucose. Instead, a significant constitutive, growth rate-associated level of *gal7* transcript was observed under all conditions tested. Induction by D-galactose was apparent, but of low efficacy (twofold). Furthermore, a major difference exists as to the specificity of the inducer: while in *S. cerevisiae* expression of *GAL7* solely depends on the presence of galactose, *H. jecorina gal7* is also induced by the pentose L-arabinose. In this context, it is interesting to note that fungal  $\alpha$ -galactosidases and  $\beta$ -galactosidases, as well as  $\alpha$ -arabinosidases, have been reported to be inducible both by D-galactose and L-arabinose, and sometimes also by D-xylose (Singh and Schügerl 1992; Nikolaev and Vientski 1998; de Vries et al. 1999). In *H. jecorina*, formation of both  $\alpha$ -galactosidase and  $\alpha$ -arabinosidase has been reported to be inducible by both D-galactose and L-arabinose, and also by the corresponding polyols (Zeilinger et al. 1993; Kristufek et al. 1994). These data, and the finding that increased levels of *gal7* expression are observed on D-galactose and L-arabinose, suggest that the natural galactose-containing carbon substrate for fungi like *H. jecorina* is likely to be polysaccharides containing both these monomers, e.g. pectins and

130

Fig. 5A–C Effect of *gal7* gene disruption on cellulase (Cel7A) synthesis and *cbh1* transcription by *H. jecorina* during growth on lactose. A Growth of *H. jecorina* QM 9414 (filled squares) and the  $\Delta gal7$  mutant (filled triangles) on lactose. B Western analysis of Cel7A protein in the culture supernatant; equal volumes (5  $\mu$ l) were loaded per lane. C Northern analysis of *cbh1* transcript accumulation in *H. jecorina* QM 9414 and the  $\Delta gal7$  mutant on lactose-containing medium. The *act1* transcript served as a loading control.



arabinogalactans (Dey and Brinson 1984). The ability of *H. jecorina* to grow on these two polysaccharides as carbon sources lends support to this hypothesis.

Galactosemia results from an inborn error of metabolism that occurs with a frequency of one patient per 47,000 in Caucasians. Any one of five amino acid exchanges in galactose-1-phosphate uridylyl transferase leads to galactose toxicity in these patients (Suzuki et al. 2001). The molecular basis of this galactose toxicity is still unclear, but it has been attributed to the accumulation of galactitol and galactose-1-phosphate (Liu et al. 2000; Ning et al. 2000). The finding of a slower growth rate and altered morphology during growth of the  $\Delta gal7$  mutant of *H. jecorina* on glucose plus galactose suggests that galactose is also toxic to *gal7*-deficient filamentous fungi. Whether this is also due to the accumulation of galactitol remains to be established, but the morphological aberrations in the mutant (swollen hyphae and thickened cell walls) would be compatible with the intracellular accumulation of a solute such as galactitol, which would increase the osmotic pressure internally. Indeed we have been able to detect the accumulation of galactitol in mycelia of the  $\Delta gal7$  mutant of *H. jecorina* during growth on lactose (unpublished data). If this is confirmed, filamentous fungi such as *H. jecorina* may serve as simple and useful models for the study of the effects of non-metabolizable galactose on eucaryotic metabolism.

Interestingly, *gal7* gene disruption did not affect the ability of *H. jecorina* to grow on lactose as a carbon source. Although it is not yet known how lactose is metabolized, i.e. whether lactose is first hydrolyzed to D-glucose and D-galactose, and these monosaccharides

then taken up and metabolized, or whether lactose is first taken up and hydrolysis occurs intracellularly, it is clear from the present data that the steps following galactose-1-phosphate uridylyltransferase have little impact on the regulation of synthesis of the respective  $\beta$ -galactosidase and that of the lactose or galactose uptake systems. One might argue that this is because synthesis of  $\beta$ -galactosidase and the permeases is constitutive; however, we have evidence that at least synthesis of  $\beta$ -galactosidase is dependent on induction. Thus the inducer is formed by a step prior to that mediated by galactose-1-phosphate uridylyltransferase, which is unaffected by the lack of this enzyme.

With respect to cellulase biosynthesis on lactose, we found that the  $\Delta gal7$  mutant accumulated the *cbh1* transcript in similar relative levels to the wild-type strain, which shows that lactose metabolism through the galactose-1-phosphate uridylyltransferase step is also not required for cellulase formation. The fact that the decreased growth rate of the  $\Delta gal7$  strain on lactose does not increase synthesis of Cel7A contrasts with the finding by several authors of an inverse correlation between growth rate and cellulase formation (Mandels and Andreotti 1978; Andreotti et al. 1980). On the other hand, the significantly delayed decay in abundance of the *cbh1*-mRNA in the  $\Delta gal7$  strain during prolonged cultivation could be due to an increased concentration or prolonged half-life of an inducer which is obviously formed prior to the Gal7 step. Taken together, these findings are more compatible with the theory that lactose acts as an inducer of cellulase formation rather than promoting cellulase biosynthesis by relieving the fungus from carbon catabolic repression. Further studies,

using mutants blocked in earlier stages of the Leloir pathway, are currently in progress.

**Acknowledgements** This study was supported by the Fifth (EC) Framework programme (Quality of Life and Management of Living Resources; Project EUROFUNG 2; QLK3-1999-00729).

## References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403-410
- Andreotti RE, Medeiros JE, Roche C, Mandels M (1980) Effects of strain and substrate on production of cellulases by *Trichoderma reesei* mutants. In: Ghose TK (ed) *Bioconversion and Bioprocessing Symposium 2. Biomedical Engineering Research Centre. Indian Institute of Technology, New Delhi*. pp 353-371
- Ausubel FM, Bront R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1990) *Current protocols in molecular biology*. Greene Publishing Associates and Wiley-Interscience, New York
- Brakhage AA, Andrianopoulos A, Kato M, Steidl S, Davis MA, Tsukagoshi N, Hynes MJ (1999) HAP-like CCAAT-binding complexes in filamentous fungi: implications for biotechnology. *Fungal Genet Biol* 27:243-252
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159
- Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 16:10881-10890
- Denison SH (2000) pH regulation of gene expression in fungi. *Fungal Genet Biol* 29:61-71
- DeVries RP, van den Broek HC, Dekkers E, Manzanares P, de Graaff LH, Visser J (1999) Differential expression of three  $\alpha$ -galactosidase genes and a single  $\beta$ -galactosidase gene from *Aspergillus niger*. *Appl Environ Microbiol* 65:2453-2460
- Dey PM, Brinson K (1984) Plant cell walls. *Adv Carbohydr Chem Biochem* 42:265-301
- Dhawal SS, Lane AC (1993) Compilation of sequence-specific DNA-binding proteins implicated in transcriptional control in fungi. *Nucleic Acids Res* 21:5537-5546
- Dickson RC, Riley MI (1989) The lactose-galactose regulon of *Kluyveromyces fragilis*. *Biotechnology* 13:19-40
- Frey PA (1996) The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J* 10:461-470
- Fukasawa T, Nogi Y (1989) Molecular genetics of galactose metabolism in yeast. *Biotechnology* 13:1-18
- Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. *Science* 274:546
- Gross KC, Phar DM (1982) A potential pathway for galactose metabolism in *Cucumis sativus* L., a stachyose transporting species. *Plant Physiol* 69:117-121
- Gross W, Schnarrenberger C (1995) Purification and characterization of a galactose-1-phosphate:UDP-glucose uridylyltransferase from the red alga *Galdieria sulphuraria*. *Eur J Biochem* 234:258-264
- Gruber F, Visser J, Kubicek CP, de Graaff LH (1990a) The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a *pyrG*-negative mutant strain. *Curr Genet* 18:71-76
- Gruber F, Visser J, Kubicek CP, de Graaff LH (1990b) Cloning of the *Trichoderma reesei pyrG* gene and its use as a homologous marker for a high-frequency transformation system. *Curr Genet* 18:447-451
- Higuchi R (1990) In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, (eds) *PCR protocols: a guide to methods and applications*. Academic Press, New York, pp 177-183
- Ilmen M, Thrane C, Penttilä M (1996) The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. *Mol Gen Genet* 251:451-460
- Johnstone IL, McCabe PC, Greaves P, Gurr SJ, Cole GE, Brow MA, Unkles SE, Clutterbuck AJ, Kinghorn JR, Innis MA (1990) Isolation and characterisation of the *crnA-niaA-niaD* gene cluster for nitrate assimilation in *Aspergillus nidulans*. *Gene* 90:181-192
- Keller NP, Adams TH (1995) Analysis of a mycotoxin gene cluster in *Aspergillus nidulans*. *SAAS Bull Biochem Biotechnol* 8:14-21
- Kristufek D, Hodits R, Kubicek CP (1994) Coinduction of  $\alpha$ -L-arabinofuranosidase and  $\alpha$ -D-galactosidase formation in *Trichoderma reesei* RUT C-30. *FEMS Microbiol Lett* 115:259-264
- Leslie ND, Yager KL, McNamara PD, Segal S (1996) A mouse model of galactose-1-phosphate uridylyl transferase deficiency. *Biochem Mol Med* 59:7-12
- Liu G, Hale GE, Hughes CL (2000) Galactose metabolism and ovarian toxicity. *Reprod Toxicol* 14:377-84
- Llois MA, Ragan MA (1998) Characterization of a galactose-1-phosphate uridylyltransferase gene from the marine red alga *Gracilaria gracilis*. *Curr Genet* 34:112-119
- Lohr D, Vcnkov P, Zlatanova J (1995) Transcriptional regulation in the yeast *GAL* gene family: a complex genetic network. *FASEB J* 9:777-787
- Mandels MM, Andreotti RE (1978) The cellulose to cellulase fermentation. *Proc Biochem* 13:6-13
- Mischak H, Hofer F, Measner R, Weissinger E, Hayn M, Tomme P, Esterbauer H, Kuchler F, Claessens M, Kubicek CP (1989) Monoclonal antibodies against different domains of cellobiohydrolase I and II from *Trichoderma reesei*. *Biochim Biophys Acta* 990:1-7
- Nikolaev IV, Vienzi YP (1998) L-Arabinose induces synthesis of secreted  $\beta$ -galactosidase in the filamentous fungus *Penicillium canescens*. *Biochemistry (Moscow)* 63:1294-1298
- Ning C, Reynolds R, Chen J, Yager C, Berry GT, McNamara PD, Leslie N, Segal S (2000) Galactose metabolism by the mouse with galactose-1-phosphate uridylyltransferase deficiency. *Pediatr Res* 48:211-7
- Penttilä ME (1998) Heterologous protein production in *Trichoderma*. In: Harman GE, Kubicek CP (eds) *Trichoderma and Gliocladium: enzymes, biological control and commercial applications*. Taylor and Francis, London, pp 365-383
- Persson I, Tjerneld F, Hahn-Hägerdal B (1991) Fungal cellulolytic enzyme production: a review. *Proc Biochem* 26:65-74
- Roberts CF (1963) The genetic analysis of carbohydrate utilization in *Aspergillus nidulans*. *J Gen Microbiol* 31:45-58
- Roberts CF (1970) Enzyme lesions in galactose non-utilizing mutants of *Aspergillus nidulans*. *Biochim Biophys Acta* 201:267-283
- Roelfsema WA, Kuster FM, Phui H (1990) Lactose and derivatives. In: Elvers B, Hawkins S, Schulz G (eds) *Ullmann's encyclopedia of industrial chemistry* (4th edn). VCH, Weinheim, pp 107-114
- Ruzicka FJ, Wedekind JE, Kim J, Rayment L, Frey PA (1995) Galactose-1-phosphate uridylyltransferase from *Escherichia coli*, a zinc and iron metalloenzyme. *Biochemistry* 34:5610-5617
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual* (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schierova M, Linka M, Pazoutova S (2000) Sulfate assimilation in *Aspergillus terreus*: analysis of genes encoding ATP-sulfurylase and PAPS-reductase. *Curr Genet* 38:126-131
- Singh A, Schügerl K (1992) Induction and regulation of D-xylose catabolizing enzymes in *Fusarium oxysporum*. *Biochem Int* 28:481-488
- Smith DJ, Burnham MK, Edwards J, Earl AJ, Turner G (1990) Cloning and heterologous expression of the penicillin biosynthetic gene cluster from *Penicillium chrysogenum*. *Biotechnology* 8:39-41



132

- Strauss J, Mach RL, Zeilinger S, Hartler G, Stöffler G, Wolschek M, Kubicek CP (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. FEBS Lett 376:103-107
- Suzuki M, West C, Beutler E (2001) Large-scale molecular screening for galactosemia alleles in a pan-ethnic population. Hum Genet 109:210-215
- Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ. (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J Bacteriol 181:6469-6477
- Van Peij NN, Gielkens MM, de Vries RP, Visser J, de Graaff LH (1998) The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. Appl Environ Microbiol 64:3615-3619
- Wedekind JE, Frey PA, Rayment I (1995) Three-dimensional structure of galactose-1-phosphate uridylyltransferase from *Escherichia coli* at 1.8 Å resolution. Biochemistry 34:11049-11061
- Wedekind JE, Frey PA, Rayment I (1996) The structure of nucleotidylated histidine-166 of galactose-1-phosphate uridylyltransferase provides insight into phosphoryl group transfer. Biochemistry 35:11560-11569
- Wickes BL, Edman JC (1995) The *Cryptococcus neoformans* GAL7 gene and its use as an inducible promoter. Mol Microbiol 16:1099-1109
- Zeilinger S, Kristufek D, Arisan-Atac I, Hodits R, Kubicek CP (1993) Conditions for formation, purification and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. Appl Environ Microbiol 59:1347-1353
- Zeilinger S, Ebner S, Marosits T, Mach RL, Kubicek CP (2001) The *Hypocrea jecorina* HAP 2/3/5 protein complex binds to the inverted CCAAT-box (ATTGG) within the *cbh2* (cellobiohydrolase II-gene) activating element. Mol Genet Genomics 266:56-63

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**